Chapter 10

Proteomic Analysis of *Caenorhabditis elegans*

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Summary

Proteomic studies of the free-living nematode *Caenorhabditis elegans* have recently received great attention because this animal model is a useful platform for the in vivo study of various biological problems relevant to human disease. In general, proteomic analysis is carried out in order to address a specific question with respect to differential changes in proteome expression under certain perturbed conditions. In this chapter, we focus on gel-based proteomic analysis of *C. elegans* subjected to two specific stress conditions during development: induction of the dauer state for whole body protein expression and a temperature shift for egg protein expression. Utilizing these differently perturbed *C. elegans* protein samples, two-dimensional electrophoresis and differential in-gel electrophoresis methods have led to the discovery of remarkable aspects of the worm's biology. We also provide numerous details about the technical points and protocols necessary for successful experimentation.

Key words: 2DE, Caenorhabditis elegans, Dauer larva, Azacoprostane, Egg proteome, MALDI-TOF, DIGE.

1. Introduction

Since *C. elegans* was established as an animal model organism in 1965 for the study of various biological problems, genomic and proteomic analyses have been used with increasing frequency to discover major aspects of its biology relevant to human disease. This is because C. elegans has many unique features that make it an ideal model organism, such as a convenient inbreeding reproductive mode (i.e., hermaphroditic), a short life span (2~3 weeks), a large brood size (>300 progeny), a variety of readily produced genetic mutants, and ease of cultivation in the laboratory *(1)*.

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After the genome of *C. elegans* had been completely sequenced, proteomics became an essential experimental strategy in analyzing global gene expression in *C. elegans* under particular physiological conditions *(2)*.

Large-scale analysis of all possible proteins expressed under specific physiological conditions, termed expression proteomics, is usually a good starting point for the study of the *C. elegans* proteome, through which one can profile those proteins of interest in a high-throughput manner. Among several proteomics platforms, we describe the most common gel-based methods such as two-dimensional electrophoresis (2DE) and differential gel electrophoresis (DIGE) for proteomic analysis of *C. elegans* obtained under two different physiological conditions (**Fig.** 1).

Firstly, we describe analysis of whole body proteins isolated from normal mixed-stage worms and dauer larvae. When nutritional and environmental conditions are adequate for growth, *C. elegans* develops rapidly from the embryo, through four larval stages

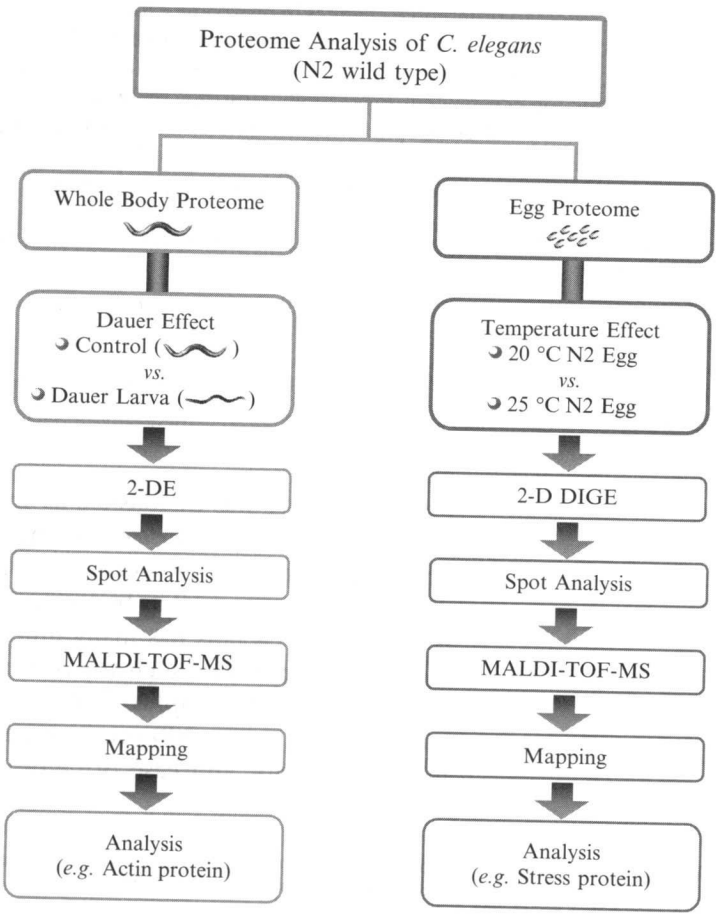


Fig. 1. Overall strategy for 2DE analysis of *C. elegans* protein.

(L1–L4), to reproductive adults. In contrast, during unfavorable conditions, *C. elegans* arrests prior to reproductive maturity, either as an L1 larva or as a specialized L3 diapause form termed the dauer larva *(3)*. In this chapter, using 2DE, we describe identification of proteins differentially expressed in dauer larvae compared to normal, mixed-stage worms. Secondly, we describe a DIGE method for the proteomic analysis of egg proteins prepared from adult *C. elegans* grown at either 20°C or 25°C. The DIGE methods were highly useful in quantifying egg proteins differentially expressed upon temperature shift, an essential part of studies of the effect of azacoprostane on *C. elegans* development. Thus, this chapter is intended to provide the reader with the necessary information for systematic analysis of the *C. elegans* proteome using 2DE or DIGE. We highlight our use of these methods to discover biomarkers involved in dauer formation and the worm's adaptation to stress caused by temperature shift.

1.1. Analysis of Whole Body Proteome of C. elegans*: Mixed-Stage Worms Versus Dauer Larvae*

To explore if there is a substantial change in protein expression between dauer larvae and mixed-stage worms, 2DE proteomic analysis was performed. **Figure 2a** is a typical 2DE gel image showing separation of proteins from mixed-stage normal worms at pH 3–10. Because protein spots in the acidic and alkaline pH areas

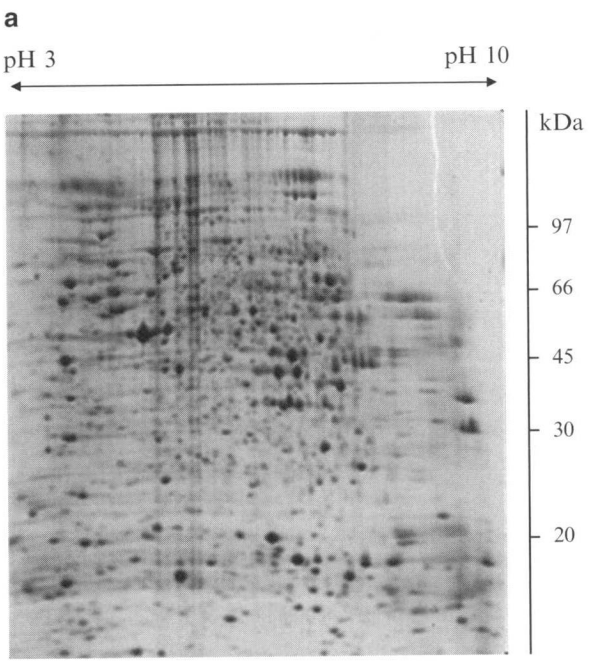


Fig. 2. 2DE gel pattern of protein extracted from mixed-stage worms and dauer larvae of *C. elegans*. 2DE gel image of proteins from mixed-stage worms at (**a**) pH 3–10, (**b**) from dauer larvae at pH 3–6, and (**c**) from dauer larva at pH 5–8. Proteins extracted from wild-type worms were separated on a nonlinear IPG strip, followed by a 9–16% SDS–polyacrylamide gel. The gel was stained with CBB.

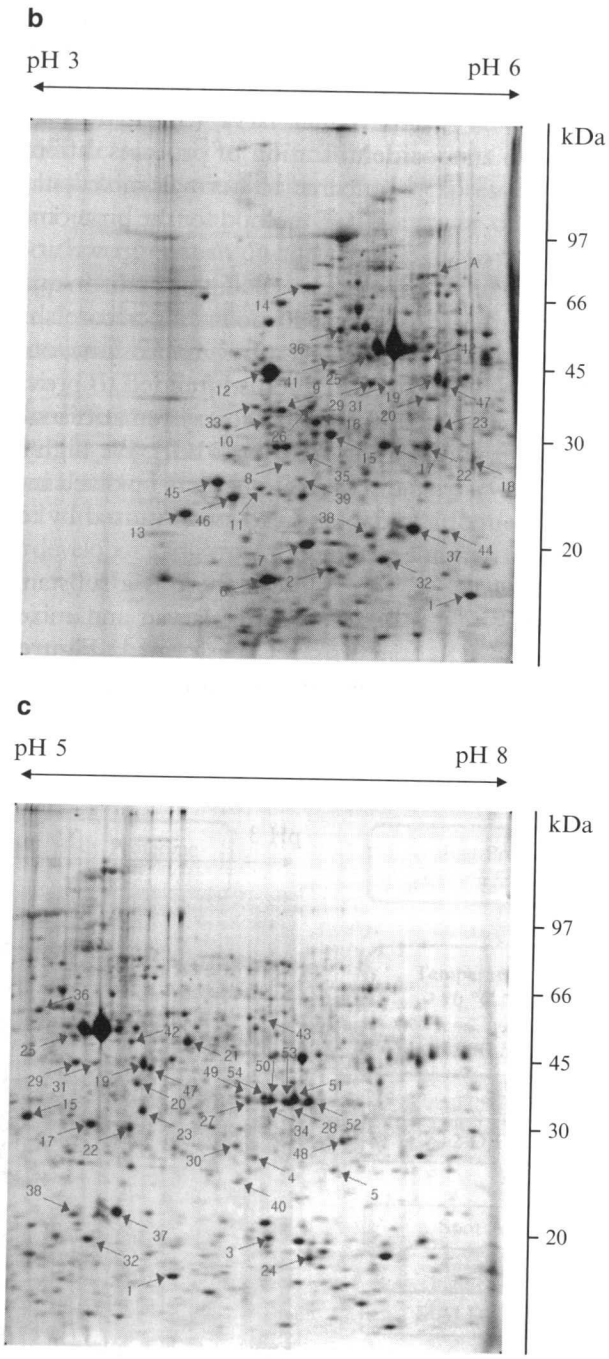


Fig. 2. (continued)

were not well resolved, whole extracts of mixed-stage worms and dauer larvae were separated on two different immobilized pH gradient (IPG) strips (i.e., pH 3–6 and pH 5–8) followed by 9–17% SDS–PAGE. Aligning spots by image analysis, we directly

compared gels representing mixed-stage worms versus dauer larvae (n = 3) and analyzed the differentially expressed protein spots in the dauers. The 2DE gel patterns of dauer larvae at pH 3–6 (**Fig. 2b**) and pH 5–8 (**Fig.** 2c) are shown. Spots in the 2DE gel were then isolated, digested with trypsin, treated with mixed POROS R2 and R3 resins, and analyzed by MALDI-TOF-MS or MALDI-TOF-MS/MS (**Subheadings 2** and **3**). Proteins were identified by mass fingerprinting of the selected peptide peaks by applying low tolerance (<20 ppm) with recalibration. The location of the corresponding peak for each protein and its mass and *pI* were also confirmed (**Table 1**). We focused specifically on protein spots that showed >2.0-fold (n = 3) intensity in dauers compared to mixed-stage worms, or proteins that were detected only in dauer larvae. These proteins were grouped into three functional categories: (1) oxidative stress-defense related proteins, (2) muscle proteins, and (3) energy generation and other proteins (**Table** 1).

Table 1
Proteins that were more abundant in dauer larvae of *C. elegans* than in mixed-stage worms

ID number[a]	Name of protein	Increase fold (n = 3) in dauer larva[b]	Accession number	Molecular mass (Da)/pI	Coverage (%)	Matched peak
Stress-defense related proteins						
1	Heat shock protein HSP-12.6	M	17541102	12621/5.6	64	5
2	Thioredoxin family member	1.387	17539056	16965/5.0	31	4
3	Superoxide dismutase [Cu–Zn]	1.302	464769	16237/6.1	52	6
4	Glutathione S-Transferase, P subunit (gst-1)	2.076	1753834	23902/5.9	29	6
5	Glutathione S-transferase GST-7	1.155	17534685	23086/6.3	25	8
Muscle structural proteins						
6	Myosin light chain (mic-3)	1.601	25151365	17145/4.6	70	12
7	Myosin light chain (mLc-2)	1.724	17569077	18603/5.1	67	9

(continued)

Table 1 (continued)

ID number[a]	Name of protein	Increase fold (n = 3) in dauer larva[b]	Accession number	Molecular mass (Da)/pI	Coverage (%)	Matched peak
8	Levamisole resistant-11, tropomyosin isoform (lev-11)	2.07	32563577	32937/4.7	37	10
9	Levamisole resistant-11, tropomyosin isoform (lev-11)	1.439	25151024	29632/4.7	47	15
10	Levamisole resistant-11, tropomyosin isoform (lev-11)	3.629	32563577	32937/4.7	44	14
11	CeTMI	M	1208409	32952/4.7	42	12
12	CeTMI	2.471	1208409	32952/4.7	33	10
13	Troponin C (pat-10)	2.44	17507581	18517/4.2	44	5
14	Troponin T TNT-2	4.987	17568063	53810/4.2	68	9
15	Actin (act-3)	4.492	6628	41709/5.3	28	8
16	Actin (act-3)	2.889	6628	41709/5.3	35	10
17	Actin (act-3)	9.424	6628	41709/5.3	27	7
18	Actin (act-3)	2.39	6628	41709/5.3	20	6
19	Actin (act-4)	M	17568987	37279/5.4	34	8
20	Actin (act-4)	M	17568987	37279/5.4	34	8
21	Actin (act-4)	9.427	17568985	41778/5.3	44	12
22	Actin (act-4)	M	17568987	37279/5.4	36	9
23	Actin (act-4)	8.462	17568987	37279/5.4	33	8
24	Actin (act-4)	M	17568987	37279/5.4	21	6
25	Actin (act-4)	1.702	17568987	37279/5.4	19	4
26	Actin (act-5)	8.517	17551718	41873/5.4	16	4
27	Annexin (nex-1)	3.213	17554342	35696/6.1	37	11
28	Annexin (nex-1)	1.138	17554342	35696/6.1	40	13
29	Disorganized muscle protein DIM-1 short isoform (dim-1)	1.507	25148955	35539/5.2	48	13
30	Disorganized muscle protein DIM-1 short isoform (dim-1)	M	25148955	35539/5.2	24	6

(continued)

Table 1
(continued)

ID number[a]	Name of protein	Increase fold (n = 3) in dauer larva[b]	Accession number	Molecular mass (Da)/pI	Coverage (%)	Matched peak
31	Disorganized muscle protein DIM-1 short isoform (dim-1)	1.867	25148955	35539/5.2	27	7
Electron transport system/energy generation proteins						
32	Cytochrome c oxidase subunit Va	1.13	17555666	20111/5.8	38	7
33	probable cytochrome P450 E03E2.1 [similarity]	M	7498337	55841/8.6	21	4
34	NADH-ubiquinone oxidoreductase, PDSW subunit (1L97)	M	17507827	31242/6.1	15	4
35	ATP synthase subunit (atp-2)	2.403	25144756	57527/5.5	24	9
36	ATP synthase subunit ATP-2	1.297	25144756	57527/5.5	48	11
Other proteins						
37	Transthyretin-like family member (4L828)	2.997	17542886	14693/5.2	34	4
38	Transthyretin-like protein precursor family member (5O10)	1.698	17559006	17777/5.8	14	3
39	Arginine kinase family member	M	32566409	39991/6.2	14	5
40	Ump-cmp kinase (2L419)	3.248	17533833	21240/5.9	37	6
41	Aspartic protease ASP-4	1.829	17549909	49278/6.1	25	6
42	4-Hydroxyphenylpyruvate dioxygenase (hpd-1)	1.885	17555220	44383/5.4	40	13
43	Homogentisate oxidase HGO-1	1.822	17507969	49239/5.9	28	7
44	Initiation factor five eIF-5A homolog IFF-2	2.017	17534327	17954/5.4	42	8
45	Allergen V5/Tpx-1 related family member (5E293)	M	17561866	22470/4.5	20	3

(continued)

Table 1 (continued)

ID number[a]	Name of protein	Increase fold (n = 3) in dauer larva[b]	Accession number	Molecular mass (Da)/pI	Coverage (%)	Matched peak
46	Allergen V5/Tpx-1 related family member (5E290)	5.484	17561870	22455/4.50	26	5
47	Ribosomal protein, small subunit (30.7 kD) (rps-0)	1.599	17554768	30703/5.5	23	6
48	Enoyl-coA hydratase	1.741	17560910	28438/6.5	26	5
49	Galectin (lec-1)	1.874	25153023	31810/6.1	24	7
50	Galectin (lec-1)	1.709	25153023	31810/6.1	36	8
51	Galectin (lec-1)	1.678	25153023	31810/6.1	28	6
52	Galectin (lec-2)	1.35	25154078	31296/6.2	29	7
53	Galectin (lec-2)	1.138	25154078	31296/6.2	34	8
54	Galectin (lec-4)	3.217	9857647	32392/6.0	27	7

M observed in the three different gels only in dauer larvae
[a]ID number indicates the protein spot in the 2DE master reference gel
[b]The mean (n= 3) factor of increase in dauer larvae compared to mixed-stage worms, obtained from the three different gels

1.2. Differentially Expressed Proteins in the Dauer Larva

1.2.1. Oxidative Stress-Defense Related Proteins

Two proteins thought to be involved in stress resistance were more abundant in dauer larvae than in mixed-stage worms: Heat shock protein-12.6 (HSP-12.6); newly detected) and glutathione transferase-1 (GST-1; 2.10-fold greater in dauers) (**Fig. 2b, c, Table 1**). Eukaryotic cells respond to heat shock by inducing a conserved set of HSPs, which act as molecular chaperones. They have the ability to prevent protein aggregation and in some cases actually promote the renaturation of unfolded polypeptides in vitro *(4, 5)*. The glutathione and thioredoxin systems represent two major antioxidant defense lines in most eukaryotes and prokaryotes. Overall, a few of these defense-related proteins are upregulated (e.g., 1.3–2.1-fold) in dauer larvae, an observation consistent with previous genomics work *(6–7)*.

1.2.2. Muscle Proteins

Several muscle proteins were detected with >2-fold upregulation or newly detected in dauer larvae: two levamisole resistant-11 proteins (2.07–3.63-fold), troponin C and T (2.44–4.98-fold), 5 ACT-3 (4.49–9.42-fold), annexin (nex-1) (3.23-fold), and DIM-1 (newly appeared) (**Table 1, Fig. 2b, c**). The increase in these

rather diverse muscle proteins might be caused by rearrangement of muscle proteins as part of a defense mechanism against oxidative stress and could indicate the presence of proteolysis in the dauer state. The slender body and specialized cuticle might also be attributed to changes in structural, muscle, and other proteins in dauers.

1.2.3. Energy Generation and Other Proteins

Dauer larvae are known to exhibit reduced energy generation and consumption *(8)*. In our experiments, ATP synthase subunit (atp-2) was increased 2.4-fold ($n = 3$) in dauers, perhaps due to decreased ATP levels. The NADH-ubiquinone oxidoreductase was newly detected in dauer larvae; this enzyme not only provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle but also couples the oxidation of NADH and the reduction of ubiquinone to the generation of a proton gradient then used for ATP synthesis. The energy supply for eukaryotic ciliary and flagellar movement is thought to be maintained by ATP-regenerating enzymes such as adenylate kinase, creatine kinase, and arginine kinase. Dauer larvae contained newly detected spots of arginine kinase, which catalyzes the reversible transfer of a phosphoryl group between a phosphorylated guanidine phosphagen and adenosine diphosphate (ADP), and allergen V5/Tpx-1 related proteins (5.48-fold). This result suggests that proteins involved in energy generation are decreased while mitochondrial oxidation increases. Dauers also possessed an increase of galectin (3.21-fold), which may lead to strengthening defense mechanisms and stabilizing cellular structure, as galectins are believed to mediate cell–cell and cell–extracellular matrix interactions during development, inflammation, apoptosis, and tumor metastasis *(9, 10)*. We also found increased UMP-CMP kinase (3.248 fold; $n = 3$) in dauer larva; this enzyme catalyzes an important step in nucleic acid synthesis: the phosphorylation of UTP, CTP, and dCTP.

1.3. Analysis of Egg Proteome by DIGE Upon Temperature Shift

C. elegans develops in a temperature-dependent manner: *ca.* 14 h are required from egg to L1 development (embryogenesis) at 20°C, but only 10 h at 25°C. Embryonic lethality at 25°C is almost three times that at 20°C (data not shown). During the course of studies on the effects of disruption of sterol biosyntheses in *C. elegans* by azacoprostane, we wondered if the expression of egg proteins would be changed when adults were perturbed by a shift in growth temperature (20–25°C). Therefore, to understand the causes of these temperature-associated differences in developmental speed and embryonic lethality, we performed proteomic analysis of eggs, which are free of *E. coli* proteins and also accurately staged from a developmental perspective. For the proteomic analysis, we searched for the most sensitive dye to detect the low-abundance proteins present in eggs; the detection limit of the CyDye used in DIGE (30–100 pg; *see* also Chapters "High-Resolution 2DE", "Two-Dimensional Difference Gel Electro-

phoresis") is much lower than conventional dyes used in 2DE such as Coomassie brilliant blue (CBB; 100 ng) and silver stain (200 pg) (*see* also Chapters "Two-Dimensional Electrophoresis (2DE): An Overview" and "Silver Staining of Proteins in 2DE Gels"). In our preliminary data, the number of proteins detected by DIGE was fourfold higher than CBB-visualized proteins in the azacoprostane-treated sample (41 vs. 168 proteins, data not shown). DIGE has other advantages over general 2DE because in 2-D DIGE the labeled samples and internal standard are then mixed and coseparated on the same 2-DE gel. Coseparation of different samples on the same gel suppresses experimental variations intrinsic to 2-DE conditions, thus enabling accurate spot detection and matching, resulting in reduction of variation between experiments *(11)*. Furthermore, the amount of proteins required for DIGE is only 1/20 (50 μg vs. 1 mg) of that needed for 2DE. Thus, we employed the DIGE system for detecting differentially expressed proteins in eggs obtained at two different temperatures. We anticipated that this experiment would provide guidance on selection of optimal experimental conditions egg proteome analysis in the presence of azacoprostane to maximize the detection of drug effects.

1.4. Egg Protein Analysis by DIGE

DIGE analysis was performed on eggs collected from F1 worms grown at either 20 or 25°C. To optimize Isoelectric Focusing (IEF), three different running conditions were evaluated (i.e., 95,000 V; 100,000 V; and 105,000 V), and then the pattern of protein spots was examined. Because 100,000 V appeared to provide the best spot resolution (**Fig. 3**), we used this condition throughout the work.

After eggs were prepared from adult N2 worms grown at 20 or 25°C, cell lysates of eggs were first labeled with Cy3 (green, for egg samples obtained from F2 grown at 20°C) or Cy5 (red, analogous samples at 25°C). An aliquot of internal pooled standard (**Subheading 3.6**) was labeled with Cy2. Each labeled sample (50 μg) was mixed and run on the 2DE gel (20 × 24 cm). The image shown in **Fig. 4a** is an overlay of Cy3- and Cy5-labeled proteins in one gel. In a separate gel (20 × 24 cm), an unlabeled preparation of the same sample (1 mg) was also run on 1DE and 2DE. This gel displayed the same pattern of spot distribution when stained with CBB. Quantitative differences between the two differentially labeled samples were assessed with an image analysis system (see also Chapter "Troubleshooting Image Analysis in 2DE"). Corresponding spots differentially detected in DIGE gels were identified by overlaying CBB stained gels, then excising these spots from the CBB-stained gel for MALDI-TOF analysis.

From these series of protein identifications by MALDI-TOF, about 55 differentially expressed spots were found, among which 26 proteins increased whereas 29 proteins decreased at

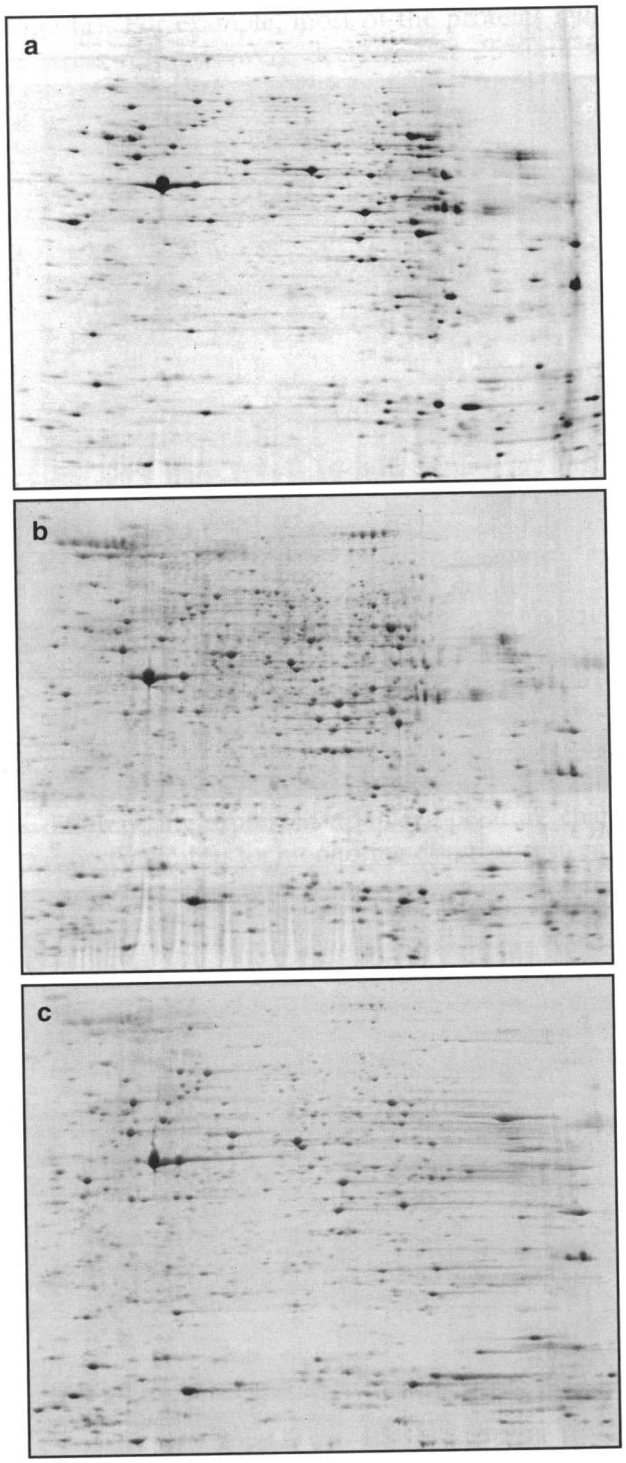
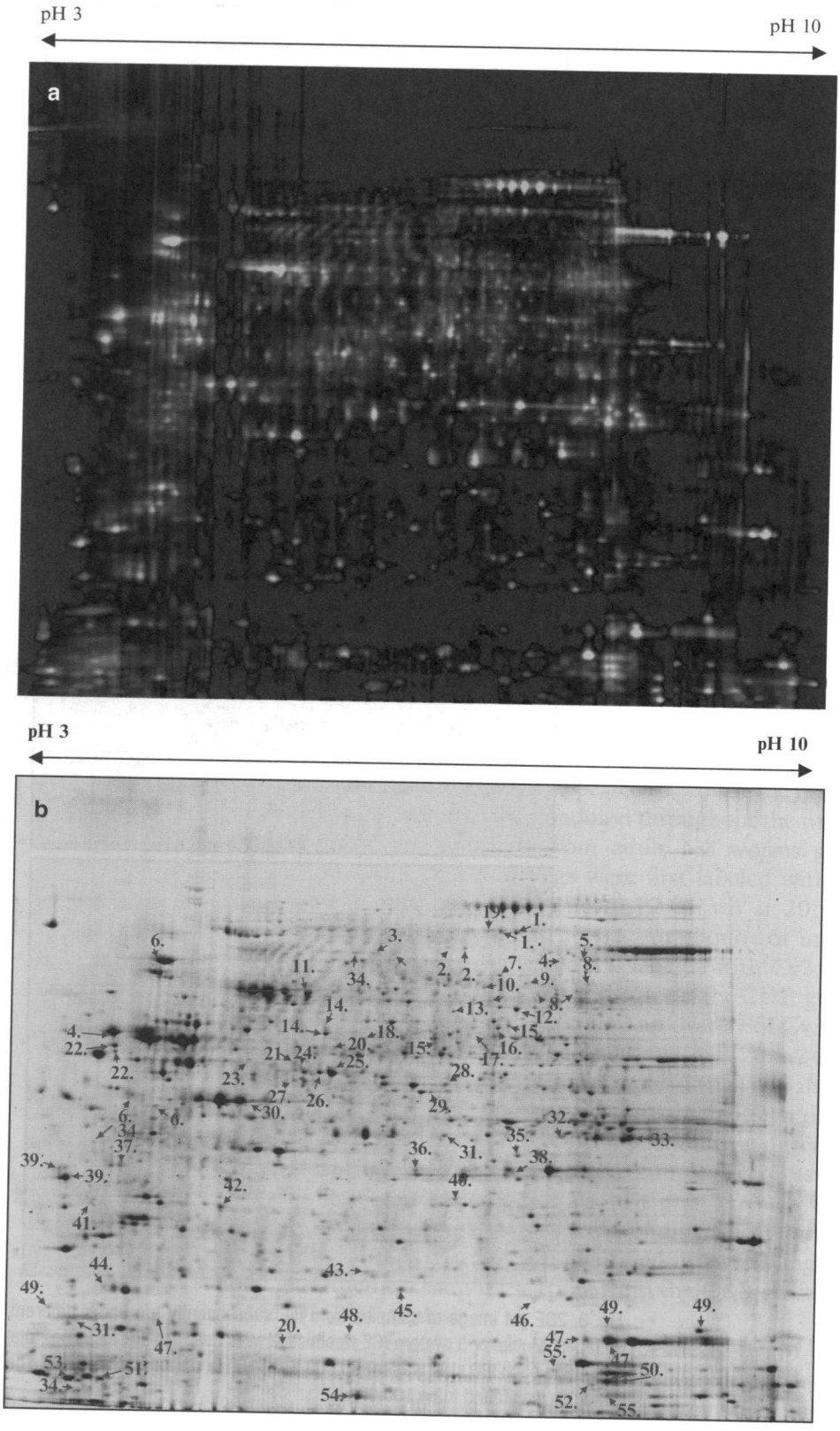


Fig. 3. 2DE gel image of samples from IEF subsequently run under different running voltages. Multiphor II system (GE Healthcare) was used for IPG focusing; total voltage applied to each IPG strip was (**a**) 95,000 V, (**b**) 100,000 V, or (**c**) 105,000 V. The optimal condition was found to be 100,000 V.


pH 3
pH 10
a
pH 3
pH 10
b

25°C (**Fig.** 4a). For example, most of the proteins involved in oxidative stress response were decreased at 25°C. They were Y71H2AM5 (cytochrome oxidase c subunit VIb), GST-7, T02H6.11 (ubiquinol–cytochrome C reductase complex 14 kD subunit), PRDX-2 (peroxiredoxin family), LET-721 (electron transfer flavoprotein-ubiquinone oxidoreductase), and UCP-4 (mitochondrial carrier protein). Chaperone proteins (e.g., DAF-21, T21B10.7, SIP-1) were also less abundant at 25°C. The consequence of this decrease in proteins involved in oxidative stress responses appears to be an observed threefold increase in embryonic lethality at 25°C as compared to 1.0% at 20°C (data not shown). Similar results in lethality and oxidative stress response protein decrease were seen in egg proteins obtained from azacoprostane-treated worms (data not shown). We also found that there is a decrease in proteins associated with protein processing (e.g., CYN-5 and -6) and protein folding, such as ribosome protein (F25H2.10, RPA-2, RPS-21). The 25°C eggs contained higher levels of proteins involved in the cell cycle, such as MCM-7, and RUVB-1, amino acid metabolism-related proteins such as K02F2.2, F01G10.1, TBH-1, WRS-1, and GRS-1, and transcription regulation protein (EFT-2). These results suggest that a temperature change from 20 to 25°C during embryonic development might cause significant perturbation in *C. elegans* metabolic pathways and cell cycle regulation. Therefore, these proteins differentially expressed upon temperature change may be used as good indicators for monitoring disturbance in embryonic development under heat stress.

Fig. 4. Effect of growth temperatures on egg protein expression. (**a**) DIGE image of egg protein sample labeled with CyDye. To identify temperature-sensitive proteins during embryogenesis, worms grown at 20°C or 25°C and egg total proteins were extracted for proteomic and DIGE analysis. 20°C-cultured N2 egg lysates were labeled with Cy3 (green), 25°C-cultured N2 egg lysates were labeled with Cy5 (red), and an aliquot of internal pooled standard was labeled with Cy2. The image is an overlay of Cy3- and Cy5-labeled proteins. (**b**) 2DE gel image of egg protein sample (used for (**a**)) stained with CBB. Differentially expressed proteins identified from twice-performed DIGE of proteins from eggs of *C. elegans* cultured at 20°C and 25°C are labeled as follows. (1) EFT (elongation factor family member), (2) MCM-7 (yeast MCM related family member), (3) hypothetical protein ZK1151.1, (4) PDI-2 (protein disulfide-isomerase (EC 5.3.4.1)) precursor (PDI-2), (5) TBB-1 (tubulin, beta family member), (6) DAF-21 (abnormal dauer formation family member), (7) GRS-1 (glycyl tRNA synthetase family member), (8) VIT-2 (vitellogenin-2 precursor), (9) hypothetical protein C01B10.8, (10) TBH-1 (tyramine beta-hydroxylase family member), (11) HSP-6 (heat shock protein family member), (12) F01G10.1, (13) LET-721, (14) T21B10.7, (15) VIT-6 (vitellogenin structural genes (yolk protein genes) family member), (16) CCT-4 (chaperonin containing TCP-1 family member), (17) ZK829.4, (18) RUVB-1 (RUVB (recombination protein) homolog family member), (19) TAG-194 (temporarily assigned gene name family member), (20) Y4C6B.1, (21) hypothetical protein C34E10.6, (22) C10G11.7, (23) T05H4.6, (24) FKB-6 (FK506-binding protein family member), (25) F57B10.3a, (26) putative RNA helicase, (27) Y71F9AL.16, (28) K02F2.2, (29) WRS-1 (tryptophanyl tRNA synthetase family member), (30) R07E5.3, (31) F43G9.1, (32) GPD-1 (GPD (glyceraldehyde 3-phosphate dehydrogenase) family member), (33) F56H6.7, (34) DYS-1 protein, (35) R09E12.3, (36) F25H2.10, (37) HIM-3 (high incidence of males (increased X chromosome loss) family member, (38) RACK-1 (RACK1 (mammalian receptor of activated C kinase) homolog family member), (39) W03D2.4, (40) NEX-1 (annexin family member), (41) C17C3.1a, (42) ZK858.3, (43) F58B3.9, (44) TCT-1 (TCTP (translationally controlled tumor protein) homolog family member), (45) PRDX-3 (peroxiredoxin family member), (46) GST-7, (47) SIP-1 (stress induced protein family member), (48) CYN-6 (cyclophylin family member), (49) CYN-5 (chain A, cyclophilin-5), (50) Y71H2AM.5, (51) F09F7.4a, (52) T02H6.11, (53) RPA-2 (ribosomal protein, acidic family member), (54) RPS-21 (ribosomal protein, small subunit family member), (55) UCP-4 (uncoupling protein (mitochondrial substrate carrier) family member).

2. Materials

2.1. Strain and Mixed N2 Culture

1. The wild-type *C. elegans* variety Bristol, N2 strain, was obtained from the *Caenorhabditis Genetics Center* (University of Minnesota, USA).
2. Wild-type *C. elegans* N2 was grown on nematode growth medium (NGM) plates and S-basal broth medium, under standard uncrowded and well-fed conditions at 20°C unless otherwise noted *(12)*.
3. Mixed N2 were isolated by washing with 0.1 M NaCl and 35% sucrose floatation and then were stored at −70°C until use.

2.2. Solutions and Buffers for C. elegans Culture (13)

1. Grow *E. coli* (OP50) in liquid culture as a food source for *C. elegans*.
2. Nematode growth medium (NGM) plate: Combine 1 L of medium containing 3 g/L NaCl, 2.5 g/L bacto-peptone, 17 g/L agar, 1 mL cholesterol stock solution (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M KH_2PO_4 (pH 6.0).
3. M9 buffer: 3 g KH_2PO_4, 6 g $Na_2HPO_4 \cdot 2H_2O$, 5 g NaCl, and distilled water to a final volume of 1 L. Adjust the buffer to pH 7.0 and autoclave. After autoclaving, add 1 mL of sterile 1 M $MgSO_4$.
4. S-basal buffer: 5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 mL cholesterol (5 mg/mL in ethanol), and distilled water to a final volume of 1 L. Sterilize by autoclaving.
5. 1 M potassium citrate (pH 6.0) solution: 20 g citric acid monohydrate, 293.5 g tri-potassium citrate monohydrate, and distilled water to a final volume of 1 L. Sterilize by autoclaving.
6. Trace-metal solution: 1.86 g disodium EDTA, 0.69 g $FeSO_4 \cdot 7H_2O$, 0.2 g $MnCl_2 \cdot 4H_2O$, 0.29 g $ZnSO_4 \cdot 7H_2O$, 0.025 g $CuSO_4 \cdot 5H_2O$, and distilled water to a final volume of 1 L. Sterilize by autoclaving and store in the dark.
7. 1 M $CaCl_2$ solution: 111 g $CaCl_2$ in 1 L of distilled water. Sterilize by autoclaving.
8. S Medium: 1 L S-basal buffer, 10 mL of 1 M potassium citrate (pH 6.0), 10 mL trace-metal solution, 3 mL of 1 M $CaCl_2$, and 3 mL of 1 M $MgSO_4$. Add components using sterile technique and do not autoclave.

2.3. Microscopy and Photography (13)

1. Microscope: Axiovert 135 (Zeiss).
2. Microcoverglass: 48 × 60 mm No. 1. (Thomas Red Label).
3. Image capture: AxioCam (Zeiss).

2.4. Isoelectric Focusing with Immobilized pH Gradient Strip (14)

1. MultiPhor™ (GE Healthcare) or Protean IEF cell (Bio-Rad): Numerous commercially available IEF units exist.
2. Reswelling tray.
3. Mineral oil: Immobiline Dry strip cover fluid (GE Healthcare).
4. Power supply, such as the EPS 3501 XL power supply (GE Healthcare).
5. Thermostatic circulator: Multitemp III thermostatic circulator (GE Healthcare).
6. IPG strips: Immobiline Dry Strip, pH 3–10 nonlinear (NL) or pH 4.0–5.0, and pH 5.5–6.7, 18 cm long, 0.5 mm thick (GE Healthcare) or with the same pH ranges for the ReadyStrip IPG strip (Bio-Rad).
7. Carrier ampholyte mixtures: IPG buffer or Pharmalyte, same range as the selected IPG strip.
8. Sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholyte, 100 mM DTT, 40 mM Tris, and a trace of bromophenol blue.
9. IPG equilibration buffer: 375 mM Tris–HCl, pH 8.8, containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 5 mM tributyl phosphine (TBP), 2.5% acrylamide solution, and 20% glycerol.
10. Agarose overlay solution: 0.5% agarose, 24.8 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS, and a trace of bromophenol blue.

2.5. Chemicals for DIGE

1. DIGE CyDye: Cy2, Cy3, Cy5 (GE Healthcare).
2. IPG strip: Immobiline Dry Strip, pH 3–10 NL, 24 cm long, 0.5 mm thick (GE Healthcare).
3. Lysis buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl, pH 8.5.
4. 2× sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 130 mM DTT, 30 mM Tris–HCl, pH 8.5.
5. Quenching solution: 10 mM lysine.

2.6. Preparation of 2DE Gels

1. Gradient former: One of the two Bio-Rad models can be used for this step: Model 385 (30–100 mL capacity) or Model 395 (100–750 mL capacity).
2. Orbital shaker with speed controller: Take care not to produce bubbles.
3. SDS–PAGE: Protean II xi multicell and multicasting chamber (Bio-Rad) or Ettan Dalt*twelve* electrophoresis system (GE Healthcare).

4. 5× Tris–HCl buffer: Dissolve 227 g Tris into 800 mL distilled water and adjust the buffer to pH 8.8 with HCl (~30 mL). Add distilled water to a final volume of 1 L.
5. 5× Gel buffer: Dissolve 15 g Tris, 72 g glycine, and 5 g SDS into 800 mL distilled water and add distilled water to a final volume of 1 L.
6. SDS equilibration buffer: Dissolve 36 g urea, 2 g SDS, 20 mL 5× Tris–HCl buffer (pH 8.8), 40 mL 50% glycerol, and 31.25 mL acrylamide monomer.
7. Acrylamide stock solution: Acrylamide/bis-acrylamide 37:5.1, 40% solution (Amresco, M157, 500 mL).
8. Fixing solution: 40% methanol and 5% phosphoric acid in distilled water.
9. CBB G-250 staining solution: 17% ammonium sulfate, 3% phosphoric acid, 34% methanol, and 0.1% CBB G-250 in distilled water.

2.7. 2DE Gel Image Analysis

1. Scanner with transparency unit, such as Bio-Rad GS710 or GS800.
2. 2DE gel image analysis program: Image Master Platinum 5 (GE Healthcare), PDQuest 7.3.0 (Bio-Rad), or Progenesis Discovery (NonLinear Dynamics, Ltd.).

2.8. Destaining and In-gel Tryptic Digestion

1. Speed Vac (Heto).
2. Sequencing-grade modified trypsin (Promega, V5111, 100 μg, 18,100 U/mg): 10 μg/mL in 25 mM ammonium bicarbonate, pH 8.0.
3. 50 mM Ammonium bicarbonate.

2.9. Desalting of Peptides and MALDI Plating

1. GELoader tips (Eppendorf, No. 0030 048.083, 20-μL capacity).
2. Poros 10 R2 resin (PerSeptive Biosystems, 1–1118–02, 0.8 g).
3. Oligo R3 resins (PerSeptive Biosystems, 1–1339–03, 6.3 g).
4. 2% formic acid in 70% acetonitrile (ACN).
5. 0.1% trifluoroacetic acid in 70% ACN.
6. 1-mL syringe.
7. Matrix: α-cyano-4-hydroxycinnamic acid (CHCA).
8. Opti-TOF™ 384-well insert (123 × 81 mm, 1016491, Applied Biosystems).

2.10. MALDI-TOF and Peptide Mass Fingerprinting

1. MALDI-TOF and MALDI-TOF/TOF: Voyager DE-Pro and 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems) equipped with a 355-nm Nd:YAG laser.
2. The pressure in the TOF analyzer is approximately 7.6e-07 Torr.

3. Methods

3.1. Nematode Culture and Sample Preparation for 2DE

1. Grow wild-type *C. elegans* (N2) at 20°C in S medium with 5 µg/mL cholesterol (Steraloids Inc, Newport, USA) using the OP50 strain of *E. coli* as a food source.
2. To prepare 2DE sample from either mixed stages or dauer larvae, grind worms in liquid nitrogen and then resuspend in an appropriate volume of sample buffer A.
3. Sonicate suspensions for approximately 240 s on ice (*see* **Note 1**), and collect the soluble fractions by centrifugation at 105,000 × *g* for 40 min at 4°C.
4. Treat with 50% trichloroacetic acid (TCA) solution.
5. Determine protein concentration in the soluble fraction by the Bradford method *(15)* using bovine serum albumin as a standard.
6. Store aliquots at –70°C until use.

3.2. Preparation of Dauer Larvae

1. Isolate dauer larvae as previously described with minor modifications *(16)* (*see* **Note 2**).
2. Grow wild-type animals in liquid culture at 20°C until food is exhausted. Then add fresh food to the medium.
3. Incubate the worms at 20°C until food is exhausted, and many dauer larvae are produced (*see* **Note 3**).
4. First isolate the worms by washing with 0.1 M NaCl and 35% sucrose floatation. Next, purify dauers by treating the worms with 1% SDS for 30 min, filtering and washing through gauze with distilled water. Store the dauers at –70°C until use (*see* **Note 4**).

3.3. Large-Volume Culture of C. elegans in NGM Broth[1]

1. Start the broth culture of *C. elegans* crowdedly cultured in the large NGM plates of N2 strain containing a mixture of adults and larvae (*see* **Note 5**).
2. Inoculate the worms and bacteria (OP50) into a fermenter containing S-basal medium.
3. Maintain the temperature at 20°C. The air flow rate is zero at the time of inoculation but after a short period of adaptation is increased to 3.0 L/min.
4. Set agitation speed at 100 rpm. When bacteria are exhausted, add OP50 again. Grow the worms continuously under this condition to produce lots of dauer larvae, even though the

[1]This Method is useful for Preparation of large quantities of protein extracts from dauer larvae

culture is not starved; dauers are produced in abundance because, when a culture is dense enough, daumone becomes a dominating influence (*see* **Note 6**).

5. When over 80% of the nematodes are dauers (an indicator of dauer pheromone), remove the worms by centrifuging in a Sorvall RC26plus centrifuge with SLA-3000 rotor for 15 min at 4,000 × *g* at 4°C.

3.4. Egg Preparation

1. Collect worms grown in S-basal medium and centrifuge them into pellets (*see* **Note 7**).
2. Suspend the worms in sterile S-basal buffer. Mix the suspension with NaOCl and 5 M NaOH in a ratio of 15: 4: 1, and then vortex the tube for a few minutes. For example, if the final solution is 1.0 mL, the ratio of volumes of each component is: S-basal with worms: NaOCl: 5 M NaOH = 750 µL: 200 µL: 50 µL. Note that worm pellets should not be too large and that the entire process should be completed within 4 min (*see* **Note 8**).
3. When about half of the adults are broken down, wash the egg-adult suspension with S-basal medium three times and then filter on a 40-µm filter. We harvest the filtered egg.
4. If it is required to synchronize to L1, add S-basal medium and continually shake the suspension. Remove salts with double-distilled water before protein extraction (*see* **Note 9**).

3.5. Egg Protein Extraction

1. Extract egg lysates directly with lysis buffer containing protease inhibitor (Complete Protease Inhibitor Cocktail, Roche).
2. Centrifuge the extracts at 35,300 × *g* for 45 min and collect supernatants.
3. Treat supernatants with 50% TCA overnight at −20°C and centrifuge at 20,000 × *g* for 20 min.
4. Wash pellets twice with 100% iced acetone and centrifuge at 20,000 × *g* for 10 min.
5. Dissolve the pellets in lysis buffer (*see* **Note 10**).
6. Adjust the pH of the protein extract to pH 8.5 by adding 50 mM NaOH (monitor with a pH indicator strip), and determine protein concentration using a 2D Quant kit (GE Healthcare).

3.6. CyDye Minimal Labeling of Egg Proteins

1. Perform CyDye labeling according to the manufacturer's (GE Healthcare) protocol (*see* **Note 11**).
2. Mix 50 µg protein sample with 400 pmole CyDye fluor by vigorous vortexing and incubate on ice in the dark for 30 min (*see* **Note 12**).
3. Label protein from purified N2 eggs at 20°C and 25°C with Cy3 and Cy5, respectively, and then mix with Cy2-labeled internal pooled standard.

4. Prepare the internal pooled standard sample by pooling 50 μg of protein from each of the two samples. Quench all labeled samples by adding 10 mM lysine for 10 min (*see* **Note 13**).
5. Suspend the total protein (150 μg) in the same volume of 2× sample buffer, and then analyze with DIGE.

3.7. 2DE of C. elegans Whole Body Proteins

1. Suspend protein samples (1.5 mg for preparative gels) in sample buffer B to obtain a final volume of 400 μL.
2. Apply aliquots of *C. elegans* proteins onto an IPG, 3–10NL (GE Healthcare), 3–6L, 5–8L (Bio-Rad) that had been rehydrated with sample protein solution at 20°C for 14 h.
3. Perform IEF at 20°C under a current limit of 50 μA/strip as follows: 100 V for 3 h; 300 V for 2 h; 1,000 V for 1 h; 2,000 V for 1 h; and then continuously at 3,500 V until reaching optimal voltage hour (Vh).
4. Perform focusing for a total of 12,000 V. Equilibrate IPG strips by gently shaking for 20 min in IPG equilibration buffer (*see* **Note 14**).
5. In the second dimension of electrophoresis, use vertical SDS gradient slab gels (9–17%; 180 × 200 × 1.5 mm).
6. Cut equilibrated IPG strips to size, and then overlay on the second-dimension gel with agarose overlay solution. Perform electrophoresis at a constant 15 mA per gel.
7. After protein fixation in 40% methanol and 5% phosphoric acid for 12 h, stain the gel with CBB G-250 overnight (*see* **Note 15**).
8. After destaining, obtain the gel image using a GS-710 image scanner (Bio-Rad).
9. Process the gel images with Melanie 4 software (GeneBio).

3.8. DIGE of C. elegans Egg Proteins

1. Rehydrate the 450 μL protein solutions (CyDye-labeled 150 μg protein for DIGE and each 1 mg protein for preparative 2DE gels) with a 24 cm Immobiline DryStrip (pH 3–10NL) in a strip holder at room temperature for 16 h, and then focus (IEF) using a MultiPhor II electrophoresis system (GE Healthcare) (*see* **Note 16**).
2. Set electrophoresis conditions at 20°C as follows – step 1: 100 V for 4 h; step 2: 300 V for 2 h; step 3: 600 V for 1 h; step 4: 1,000 V for 1 h; step 5: 2,000 V for 1 h; step 6: 3,500 V for 27 h.
3. After IEF, subject IPG strips to a one-step reduction and alkylation by TBP-equilibration buffer for exactly 25 min.
4. Apply the strips to second-dimension 9–16% SDS–PAGE gels using an Ettan Dalt*twelve* electrophoresis system (GE Healthcare).
5. Electrophorese at 20°C as follows: 2.5 W/gel for 30 min, then 16 W/gel for 6 h. For 2DE analysis, IEF/SDS–PAGE is

performed using 1 mg of each sample; the gel is stained with CBB and then scanned.

6. Perform all procedures in the dark (*see* **Note 17**).

3.9. DIGE Image Analysis (CyDye Labeling)

1. Perform spot matching and statistical analysis using DeCyder BVA (Biological Variance Analysis) v6.5.11 (GE Healthcare) (*see* also Chapter "Troubleshooting Image Analysis in 2DE").
2. Set the estimated number of spots to 3,500.
3. Group each gel into "standard," "20dgr N2," or "25dgr N2" to allow comparison between the different gels.
4. Accept only statistically significant spots ($p < 0.05$).
5. Filter the accepted spots based on an average volume ratio of 1.5-fold.

3.10. Image Scan and Acquisition

1. Visualize the CyDye-labeled gels using a Typhoon 9400 imager (GE Healthcare) and scan according to excitation/emission wavelengths for each DIGE fluor: Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm) (*see* **Note 18**).
2. Capture images and select areas of interest with ImageQuant v2005 (GE Healthcare).
3. In the case of preparative gels for protein identification, stain with CBB.

3.11. In-Gel Digestion of Protein Spots from 2DE and Protein Identification by MALDI-TOF-MS

1. Excise gel spots with end-removed pipette tips to accommodate various spot diameters.
2. Destain the gel slice in the microtube and dehydrate with 50 µL acetonitrile for 5 min at room temperature.
3. Rehydrate dried gels with 10 µL trypsin solution for 45 min on ice.
4. After removing the excess solution, digest proteins in the gels at 37°C for 24 h.
5. Treat the peptide mixtures thus obtained with the resin solution (POROS R2:Oligo R3 = 1:1 in 70% acetonitrile) *(17)*.
6. Analyze the peptides with a 4800 MALDI-TOF mass spectrometer (Applied Biosystems) operated in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV and sum data from 1,000 laser pulses.
7. Mix approx. 0.5 µL of α-cyano-4-hydroxycinnamic acid with the same volume of sample. Use the following parameters for time-of-flight measurement: 20 kV accelerating voltage, 75% grid voltage, 0% guide wire voltage, 120 ns delay, and low mass gate of 500 Da. Perform internal calibration also using

autodigestion peaks of porcine trypsin ($[M + H]^+$, 842.5090, and 2211.1064).

8. Analyze the peptide mass profiles produced by MALDI-TOF-MS using search programs such as MS-Fit 3.2 provided by UCSF (http://prospector.ucsf.edu) and ProFound (Version 4.7.0) provided by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe) with the NCBI database. Use a mass tolerance of 20 ppm for masses measured in reflectron mode.

3.13. MALDI-TOF/TOF-Mass Spectrometry

1. Use an Applied Biosystems 4800 Proteomics Analyzer (Applied Biosystems, Framingham. MA).
2. This TOF/TOF instrument is equipped with an Nd:YAG laser with 355-nm wavelength of <500-ps pulse and 200-Hz firing rate.
3. In both MS and MS/MS mode, the TOF/TOF operates at a 200 Hz repetition rate.
4. To attain such a high repetition rate, the high-voltage source 1 pulser circuit is modified.
5. The instrument uses a two-stage mirror for better focusing across the broad energy range of fragments in the MS/MS mode, without sacrificing performance in the MS-only mode.
6. Collision energy in the TOF/TOF instrument is defined by the potential difference between the source acceleration voltage and the floating collision cell, while this voltage difference has been set as 1 or 2 kV. Air is used as a collision gas at pressures of 1.5×10^{-6} Torr and 5×10^{-6} Torr.
7. All fragments are formed from the ion of interest in this region. MS/MS data are further processed using DataExplorer 4.0 (Applied Biosystems, Framingham, MA).
8. Calibrate the spectrum with the tryptic autodigested peaks (m/z 842.5090 and 2211.1046), and monoisotopic peptide masses obtained with Data Explorer 3.5 (PerSeptive Biosystems).
9. Search the Swiss-Prot and NCBInr databases with the Matrix Science (http://www.matrixscience.com) search engine.

3.13. 2D Gel Image Analysis

1. Import the gel image (a 12–16 bit tiff file format is recommended) and convert to an ImageMaster file (*.mel).
2. Detect the protein spots and determine the volume and the % volume of each spot. The % volume is the normalized value that remains relatively independent of any irrelevant variations between gels, particularly those caused by varying experimental conditions.
3. Select differentially displayed protein spots.

4. Notes

1. *Prevention of heat generation*: Sonicate for 30 s, cool for 60 s, and pulse (50% reduced) to prevent heat generation.
2. *Prevention of contamination*: Prevent any contamination from yeast or fungi. Once contaminated, dauer formation frequency is drastically reduced or worms are dead.
3. *Dauer larvae preparation*: It would be better to add excessive amounts of food to increase the population. Additional food can be supplied when food is exhausted, which leads to an increase in the population that results in dauer formation (>80%). If food is not adequate, arrested worms at the L1 stage can be seen.
4. *Filtration*: Purified dauer populations were isolated by filtering through 40-μm Falcon cell strainers after 1% SDS treatment. This procedure should be done at room temperature to prevent SDS precipitation.
5. *Inoculation of C. elegans to the large broth culture*: About 2 mL of a suspension of worms collected in large NGM plate are inoculated into fermenter containing 6 L culture broth.
6. *Long-term dauer population*: When >80% of worm population reach the dauer state, worms are cultured 5 more days to obtain homogeneous long-term dauers.
7. *Preparation of C. elegans eggs*: Newly collected eggs are washed with double distilled water to remove any residual salts. This is very important because S-basal medium contains usually high salt levels that might cause streaking problems in 2DE gel analysis. To separate eggs from worm debris, filter the pellets through a 40-μm Falcon cell strainer, which allows eggs to pass through (*see* also **Note 4**). When *C. elegans* is prepared at different stages, separation on a sucrose gradient removes L4 or adults. Also, L1–L3 worms can pass through a10-μm-pore diameter sieve, which also removes any worms larger than these stages.
8. *Problem of bacterial contamination*: If worms are contaminated with bacteria, the effect of NaOCl is reduced. In this case, centrifuge at 800 × *g* for 1 min to remove residual bacteria. If worms are exposed to NaOCl for a longer time, egg hatching will be substantially decreased.
9. *Sample preparation*: It is also important to remove salts for many good reasons in the process of 2DE and DIGE. Residual salts may cause a shift in protein migration in IEF or horizontal streaking phenomena in the gel image (see also Chapters "Two-Dimensional Electrophoresis (2DE): An Overview and "Solubilization of Proteins in 2DE: An

Outline"). Among the many methods of salt removal (e.g., ultrafiltration, gel filtration, solid-phase extractions, dialysis, etc.), TCA precipitation was found to be the most efficient way to remove salts. This method is quick and removes other debris (e.g., lipids, polysaccharides, nucleic acids) that might adversely affect gel separation. When TCA precipitation is performed, the resulting pellets should be washed with 100% iced acetone.

10. *TCA removal*: Any residual TCA remaining in the sample may cause an acidic change in pH of the sample, thereby necessitating use of greater amounts of the NaOH solution to adjust pH to 8.5 for CyDye labeling. After washing the sample with acetone, samples can be dried no longer than 5 min. Additional drying may affect the solubility of sample pellets. It is desirable to centrifuge the sample at 20,000 × *g* for 20 min in order to remove any insoluble proteins in the sample. After immediately mixing the protein extracts, they should be divided into aliquots and frozen at −80°C until use, when they should be thawed on ice. HPLC-grade water should be used to make the lysis buffer.
11. *Sample labeling using CyDye fluors*: Each CyDye should be dissolved in dimethylformamide (DMF) to create a stock solution of 400 pmole/μL. It is also important to maintain the ratio between protein (50 μg) and CyDye fluor (400 pmol). For the best quality of CyDye, use fresh DMF (<2 month after opening cap). Because CyDye is very sensitive to light, perform all sample labeling procedures in the dark.
12. *Addition of dye*: Add the dye to the walls of reaction tubes to make sure that the two labeling reactions initiate at the same time. When adding the dye, mix completely by repeating vortexing and centrifuging several times.
13. *Quenching*: Quenching solutions also should be added to the walls of reaction tubes. After quenching, labeled samples should be loaded immediately onto the gels. If this is not possible, stop the reaction before mixing the two-dye reaction mixture and freeze the samples immediately after quenching.
14. *Storage of equilibrated IPG strip*: After IEF, the strip should be equilibrated immediately to prevent diffusion and loss of protein. An equilibrated strip can be stored for several weeks at −80°C until use.
15. *Coomassie Brilliant Blue G-250 Staining (13)*: Fix the separated proteins into the gel in 200 mL fixing solution for 1 h. Decant the fixing solution and stain the gel in CBB G-250 overnight. Decant the staining solution. Wash several times (>3 times) in distilled water for more than 4 h. Scan the gel, then wrap the gel in plastic, and store at 4°C.

16. *pH range of strip and gel gradient*: We used 3–10 nonlinear IPG strips to obtain clearly displayed spot images when proteins were separated on the gel on 9–17% gradient SDS–PAGE.
17. *Rehydration and IPG strip focusing*: There are two methods for sample rehydration: one is a cup-loading method in which the strip is soaked in sample buffer only and rehydrated after loading the sample in cup. The other method is an in-gel based rehydration method, in which the strip is soaked with the sample buffer and labeled sample. We use the in-gel based rehydration method because the cup-loading method often creates vertical smears around the cup-loading portion in the gel image. In the case of *C. elegans* egg proteins, optimal voltage was 100,000 V (**Fig. 2**).
18. *DIGE Image acquisition*: After the second-dimensional separation, gel plates can be directly scanned by Typhoon 9400 (no removal of gels from the plate is necessary). Images scanned by Typhoon 9400 can then be edited by the ImageQuant program, in which we usually crop the size of the gel image and spot position in a parallel manner because well-cropped images facilitate spot matching and analysis in DyCyder software (*see* Chapter "Immunoblotting 2DE Membranes").

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References

1. Riddle, D., and Albert, P. (1997) *C. elegans II* (Riddle, D., Blumenthal, T., Meyer, B., and Priess, J., eds.), Cold Spring Harbor Laboratory Press, pp. 739–768
2. Paik, Y. K., Jeong, S. K., Lee, E. Y., Jeong, P. Y., and Shim, Y. H. (2006) *C. elegans*: an invaluable model organism for the proteomics studies of the cholesterol-mediated signaling pathway. *Expert Rev Proteomics* 3, 439–453
3. Cassada, R. C., and Russell, R. L. (1975) The dauer larva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Dev Biol* 46, 326–342
4. Horwitz, J. (1992) α-Crystallin can function as a molecular chaperone. *Proc Natl Acad Sci U S A* 89, 10449–10453
5. Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998) The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J Biol Chem* 273, 11032–11037

6. Holt, S. J., and Riddle, D. L. (2003) SAGE surveys *C. elegans* carbohydrate metabolism: evidence for an anaerobic shift in the long-lived dauer larva. *Mech Ageing Dev* 124, 779–800
7. Wang, J., and Kim, S. K. (2003) Global analysis of dauer gene expression in *Caenorhabditis elegans*. *Development* 130, 1621–1634
8. Wadsworth, W. G., and Riddle, D. L. (1989) Developmental regulation of energy metabolism in *Caenorhabditis elegans*. *Dev Biol* 132, 167–173
9. Raz, A., Zhu, D. G., Hogan, V., Shah, N., Raz, T., Karkash, R., et al. (1990) Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis. *Int J Cancer* 46, 871–877
10. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) Apoptosis of T cells mediated by galectin-1. *Nature* 378, 736–739
11. Alban, A., David, S. O., Bjorkesten, L., Andersson, C., Sloge, E., Lewis, S., et al. (2003) A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 3, 36–44
12. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94
13. Choi, B. K., Shin, Y. K., Lee, E. Y., Jeong, P. Y., Shim, Y. H., Chitwood, D., and Paik, Y. K. (2008) Proteomic analysis of the sterol-mediated signaling pathways in *C. elegans*. *Methods Mol Biol*, Humana Press, 462 (11), 167–179
14. Cho, S. Y., Lee, E. Y., Kim, H. Y., Kang, M. J., Lee, H. J., Kim, H., *et al.* (2008) Protein profiling of human plasma samples by two-dimensional electrophoresis. *Methods Mol Biol*, Humana Press, 428, 57–75
15. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254
16. Epstein, H. F., and Shakes, D. C. (1995) *Caenorhabditis elegans*: modern biological analysis of an organism. In: *Cell Biology*, Academic Press, London
17. Larsen, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M., and Roepstorff, P. (2001) Phosphoproteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* 1, 223–238